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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/825,872	04/05/2001	Alan Solomon	044137-5029-US	3133

9629 7590 11/08/2007

MORGAN LEWIS & BOCKIUS LLP
1111 PENNSYLVANIA AVENUE NW
WASHINGTON, DC 20004

EXAMINER
KAM, CHIH MIN

ART UNIT	PAPER NUMBER
1656	

MAIL DATE	DELIVERY MODE
11/08/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	09/825,872	SOLOMON ET AL.	
	Examiner	Art Unit	
	Chih-Min Kam	1656	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *29 August 2007*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,32-34,39-45,50-52,57-61 and 63-70* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☒ Claim(s) *1,32-34,39-45,50-52,57,58,63,69 and 70* is/are allowed.

6)☒ Claim(s) *59-61 and 65* is/are rejected.

7)☒ Claim(s) *64 and 66-68* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

1. The Request for Continued Examination (RCE) filed on August 29, 2007 under 37 CFR 1.114 is acknowledged. An action on the RCE follows.

Status of the Claims

2. Claims 1, 32-34, 39-45, 50-52, 57-61 and 63-70 are pending.

Applicants' amendment filed August 29, 2007 is acknowledged. Applicants' response has been fully considered. Claims 1, 3-34, 39, 50 and 58-59 have been amended, claims 2 and 37-38 have been cancelled, and new claims 69 and 70 have been added. Therefore, claims 1, 32-34, 39-45, 50-52, 57-61 and 63-70 are examined.

Withdrawn Claim Rejections - 35 USC § 112

3. The previous rejection of claims 1-2, 32-34, 37-45, 50-52, 57-61 and 63-68 under 35 U.S.C. 112, first paragraph, scope of enablement, is withdrawn in view of applicants' amendment to the claims, applicants' cancellation of the claims, and applicants' response at pages 6-10 of the amendment filed August 29, 2007.

Withdrawn Claim Rejections - 35 USC § 102

4. The previous rejection of claims 1, 2, 32-34, 37-45, 50-52, 57-61 and 63-68 under 35 U.S.C. 102(e) as being anticipated by Schenk (U.S. Patent 6,875,434), is withdrawn in view of applicants' amendment to the claim, , applicants' cancellation of the claims, and applicants' response at page 10 in the amendment filed August 29, 2007.

5. The previous rejection of claims 1, 32-34, 37-45, 57, 59 and 64-68 under 35 U.S.C. 102(a) as being anticipated by Schenk (WO 99/27944), is withdrawn in view of

applicants' amendment to the claim, applicants' cancellation of the claims, and applicants' response at pages 10-11 in the amendment filed August 29, 2007.

New Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.

6. Claims 59-61 and 65 are rejected under 35 U.S.C. 102(a) as being anticipated by Wall *et al.* (Methods in Enzymology 309, 204-219 (1999)) as evidenced by Soloman *et al.* (US 2002/0019335).

Wall *et al.* teaches agitation-stimulated fibrillogenesis of immunoglobulin light chain peptides, recombinant V_L fragments and whole Bence Jones proteins to produce a 1 mg/ml of fibril solution in phosphate-buffered saline (pages 206-208, 212-214; claims 59-61 and 65). Since the reference teaches the fibril solutions of immunoglobulin light chain peptides having 1 mg/ml (i.e., an effective amount), which is the concentration used for immunization as evidenced by Soloman *et al.* (US 2002/0019335; paragraphs [0159], [0160]).

Claim Objections

7. Claims 64 and 66-68 are objected to because the claims are dependent from a rejected claim.

Conclusion

8. Claims 59-61 and 65 are rejected; and claims 64 and 66-68 are objected to. It appears that claims 1, 32-34, 39-45, 50-52, 57-58, 63 and 69-70 are free of art.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Chih-Min Kam whose telephone number is (571) 272-0948. The examiner can normally be reached on 8.00-4:30, Mon-Fri.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Kathleen Bragdon can be reached at 571-272-0931. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Chih-Min Kam, Ph. D.
Primary Patent Examiner

CHIH-MIN KAM
PRIMARY EXAMINER

CMK
November 5, 2007

Methods in Enzymology 309, 204–217 (1999)

DMSO) is recommended at 0–5° for brief 1- or 2-week intervals. Complete removal of the organic solvent should be done prior to dissolution in aqueous solution.

3. All solvents should be of the highest purity level and virtually free of all metal contaminants. If possible, all glassware should be rinsed with EDTA solution and deionized water before use.
4. Analysis of the D-Asp content of the Aβ may not be required. Instead, before purchasing the Aβ from a commercial source, request information about the coupling reagents that were employed during the peptide synthesis. If more reliable reagents such as HATU were used, then the possibility of racemization is not serious.

With a greater appreciation of the purity content and consistency with handling the Aβ, the end result is an enormous savings in time and money, which in turn may hasten the development of a useful therapeutic inhibitor of Aβ-amyloid formation for the treatment of AD patients.

Acknowledgments

Supported in part by grants from the National Institutes of Health (AG-08992-06 and AG-14363-01), Philip Morris, Inc., the Smokeless Tobacco Research Council, and a Faculty Scholars Award from the Alzheimer's Association to M.G.Z (FSA-94-040). The 600-MHz NMR spectrometer was purchased with funds provided by the National Science Foundation, the National Institutes of Health, and the state of Ohio. We also thank Witold Surewicz (CWRU), Nanda Menon (University of Georgia), Beth Neuhaus (University of Georgia), and Mark Smith (CWRU) for useful comments, Shu Guang Chen (CWRU) for analysis of the Aβ by MS, Harry LeVine (Parke-Davis, Inc.) and Gregory Cole (UCSD) for providing preliminary data about the Aβ fibril deposition in DMSO solution, and finally Russel Rydel (Athena Neurosciences, Inc.) for suggesting the phrase "the peptide from hell."

[14] *In Vitro* Immunoglobulin Light Chain Fibrillogenesis

By JONATHAN WALL, CHARLES L. MURPHY, and ALAN SOLOMON

Amyloid: An Introduction

Amyloidosis is a disease state resulting from the aggregation and deposition of normally soluble, innocuous proteins as insoluble fibrils. At present, more than 17 precursor proteins have been associated with amyloid syndromes. Irrespective of the type of precursor protein, however, amyloid deposits *in vivo* share a remarkable number of common structural and

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compositional similarities.[1] The precise mechanism of amyloidogenesis *in vivo* remains enigmatic. When resolved, the process will undoubtedly involve a number of accessory macromolecules in addition to the amyloid precursor protein itself, as well as the occurrence of favorable physiological factors. The assistance of chaperones or accessory molecules during amyloid formation *in vivo* is inferred by the ubiquitous presence of proteins such as P-component, glycosaminoglycans, and apolipoproteins.[2,3] How these accessory molecules initiate, perpetuate, or stabilize fibril formation, however, remains unclear. What is certain is that they are not mandatory requirements for fibril formation, implying that the amyloidogenic potential of a precursor protein resides in part within the protein itself.[4]

All amyloid deposits are clinically identified according to the following three criteria: (1) The appearance of unbranching, linear fibrils of approximately 7–10 mm in diameter and of variable length when viewed by electron microscopy; (2) binding of Congo red, resulting in a hyperchromic shift in the absorbance spectrum and blue-green birefringence when viewed microscopically using cross-polarizing filters[5]; and (3) green fluorescence on addition of the benzothiazole dye, thioflavin T (ThT), using excitation and emission wavelengths of 450 and ~485 nm respectively.[6,7] These characteristics are also used for the experimental investigation of *in vitro* fibrillogenesis.[8,9]

Immunoglobulin Light Chain Aggregation

Immunoglobulin light chain (IgLC), primary amyloidosis (AL) is an invariably fatal, systemic syndrome resulting from the overproduction and fibrillogenesis of light chain proteins, generally resulting in multiple organ dysfunction.[10,11] The major form IgLC protein associated with AL amyloid is the N-terminal 110 amino acids comprising the light chain variable region,

[1] J. D. Sipe, *Crit. Rev. Clin. Lab. Sci.* **31,** 325 (1994).
[2] M. B. Pepys, D. R. Booth, W. L. Hutchinson, J. R. Gallimore, P. M. Collins, and E. Hohenester, *Amyloid Int. J. Exp. Clin. Invest.* **4,** 274 (1997).
[3] J. H. Magnus and T. Stenstad, *Amyloid Int. J. Exp. Clin. Invest.* **4,** 121 (1997).
[4] M. Schiffer, *Am. J. Pathol.* **148,** 1339 (1996).
[5] H. Puchtler, F. Sweat, and M. Levine, *J. Histochem. Cytochem.* **10,** 355 (1962).
[6] H. Naiki, K. Higuchi, M. Hosokawa, and T. Takeda, *Anal. Biochem.* **177,** 244 (1989).
[7] H. Levine, *Amyloid Int. J. Exp. Clin. Invest.* **2,** 1 (1995).
[8] H. Naiki, K. Higuchi, K. Nakakuki, and T. Takeda, *Lab. Invest.* **65**(1), 104 (1991).
[9] L. R. Helms and R. Wetzel, *J. Mol. Biol.* **257,** 77 (1996).
[10] A. Solomon and D. T. Weiss, *Amyloid Int. J. Exp. Clin. Invest.* **2,** 269 (1995).
[11] C. A. Vaamonde, G. O. Perez, and V. Pardo, *in* "Diseases of the Kidney" (R. W. Schrier and C. W. Gottschalk, eds.), p. 2189. Little, Brown and Co., Boston, 1992.

V_L. In addition, nonfibrillar, "amorphous" aggregates may also form in patients with monoclonal gammopathies. Amorphous aggregates of IgLCs often deposit in the kidney and exhibit neither microscopic nor spectroscopic properties of amyloid fibrils. Two pathologies are distinguished based on the distribution of these kidney deposits: Light chain deposition disease characterized by glomerular precipitates and cast nephropathy associated with renal tubular deposits. Furthermore, crystalline deposits have also been observed in the renal tubules and, rarely, within the urine itself.[12] No clinical evidence exists to suggest that all three types of aggregate can be formed from the same protein in an individual patient. However, they appear not to be mutually exclusive, as both casts and amyloid have been documented in a single patient. The same protein was implicated in both aggregates by N-terminal, amino acid sequence analysis.[13,14] We suggest that the three aggregate types represent competitive pathways down which a susceptible protein may travel. Ultimately, the resultant aggregation will depend on kinetic factors, favorable associations with host macromolecules, and the microenvironment of the proteins, as well as the inherent proclivity of the protein itself, related to its primary structure.[10]

This article describes techniques specifically for managing and monitoring the production of synthetic fibrils derived from IgLC peptides V_L, proteins, and whole Bence Jones proteins (BJps). These *in vitro* techniques have been developed in order to minimize complications arising from amorphous aggregation, thus allowing the investigation of the underlying biochemical and physical factors governing light chain fibrillogenesis.

Agitation-Stimulated Fibrillogenesis

This approach has been applied successfully to the fibrillogenesis of peptides, recombinant V_L fragments, and whole BJps. Initial experiments used a 24-mer peptide as the fibril precursor, originally isolated as fibrils from a tryptic digest of the κ1 Bence Jones protein isolated from patient WAT. The peptide constitutes the N-terminal 24 amino acid residues in framework region one (FR I), which was synthesized by standard F-moc procedures. The sequence of two peptides used extensively as a fibril precursor in the agitation-stimulated fibrillogenesis (ASF) system follows:

[12] A. Solomon, D. T. Weiss, and A. A. Kattine, *N. Engl. J. Med.* **324,** 1845 (1991).
[13] M. B. Stokes, J. Jagirdar, O. Burchstin, S. Kornacki, A. Kumar, and G. Gallo, *Mod. Pathol.* **10,** 1059 (1997).
[14] B. Kaplan, R. Vidal, A. Kumar, J. Ghiso, B. Frangione, and G. Gallo, *Clin. Exp. Immunol.* **110,** 472 (1997).

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                  1          10          20
WAT(1–24)     DIQMTQSPSSLSASVGDRVTITCR-
WAT(13–24)                  ASVGDRVTITCR-
```

The 12-mer designated WAT(13–24) has also been tested thoroughly and found to form fibrillar aggregates readily using this technique (Fig. 1).

The standard protocol employed in our laboratory for the production of fibrils is as follows. A 1-mg/ml^{-1} solution of protein/peptide (used interchangeably hereafter) is prepared in phosphate-buffered saline (PBS) using high-performance liquid chromatography (HPLC)-grade water and adjusted to pH 7.5. The solution is passed through a 0.2-μm pore-sized filter to remove preformed aggregates. A 1-ml volume of this solution is placed in a 10-ml volume glass test tube and placed in a thermostatted orbital shaker (Queue Orbital Shaker, Parkersburg, WV). The protein solution is agitated at 37° and 225 revolutions per minute. Depending on the type of protein substrate used, solution turbidity can be detected within 1–10 days (Fig. 2). Peptides WAT(1–24) and WAT(13–24) generally form precipitates within 24 hr, V_L fragments may require up to 5 days, and whole BJps aggregate within 3–10 days (Fig. 2). There are, however, exceptions to these generalities, as certain V_L proteins may require extensive shaking; 21 days is the longest time observed to date.

ASF has been used to monitor both the kinetics and the equilibrium concentration of fibrillar material in solution. The kinetics may be accessed by routine sampling of the mixture over the course of the experiment. Samples are removed from the shaker and a 200-μl aliquot is placed in

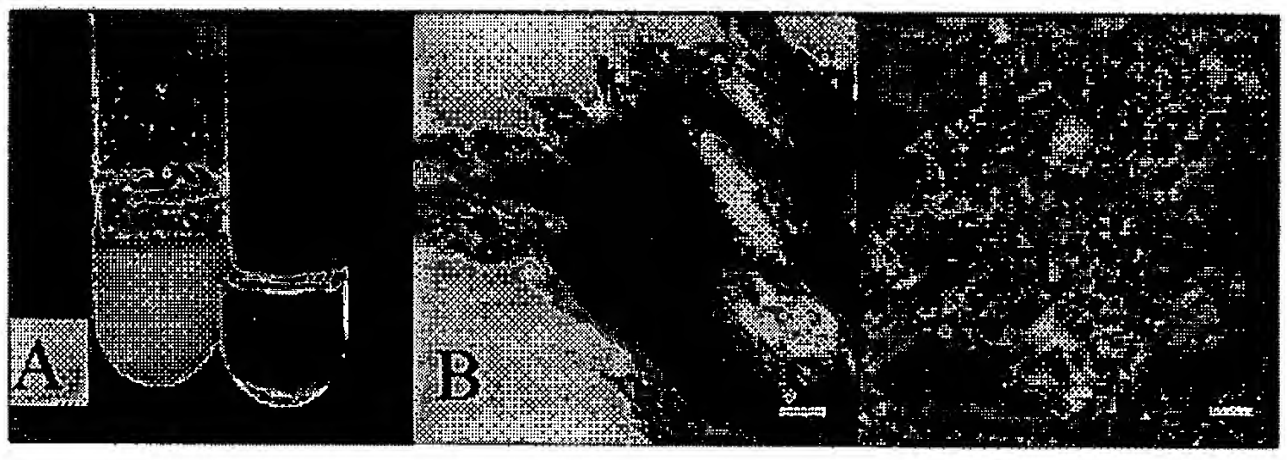


FIG. 1. Production of amyloid fibrils by ASF in an orbital shaker. (A) Test tubes showing the formation of fibrils by ASF using 1 mg/ml of V_L (left) and control protein (right). (B) Negatively stained, electron microscopy of WAT(1–24) fibrils formed by ASF. Bar: 120 nm. (C) Negatively stained, electron microscopy of V_L fibrils formed by ASF. Bar: 90 nm.

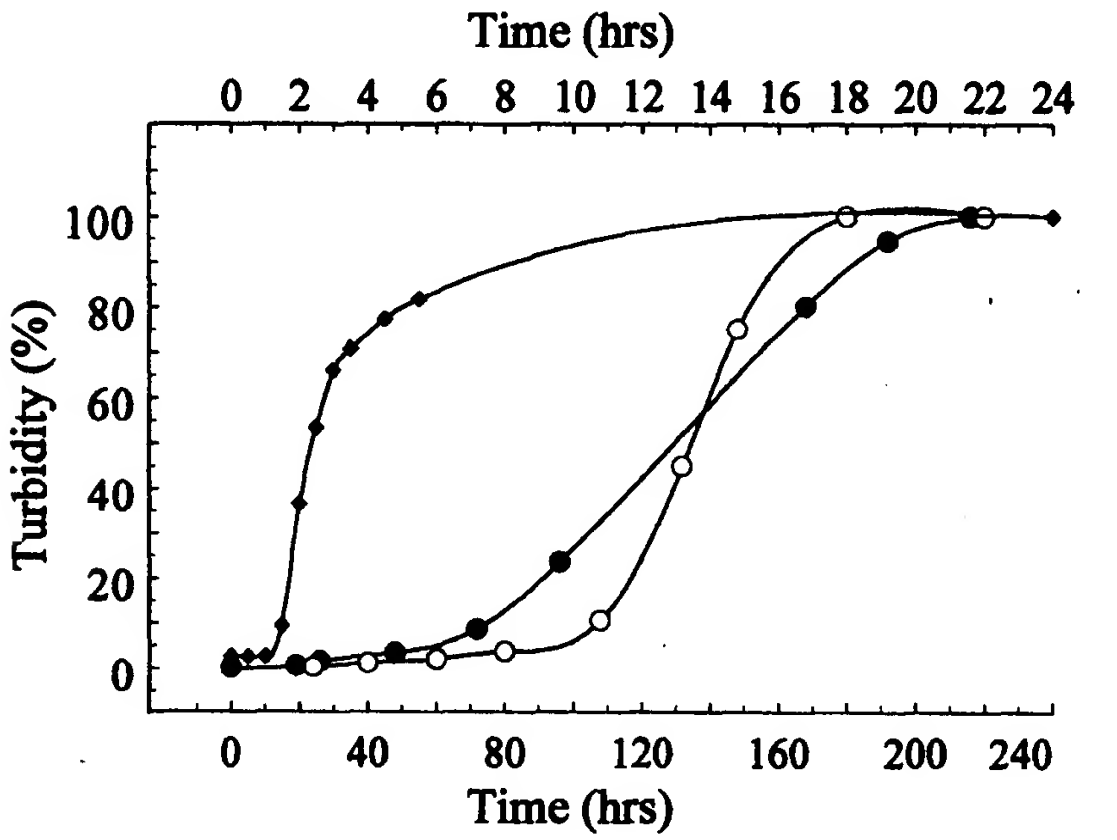


FIG. 2. Agitation-stimulated fibrillogenesis. Changes in the turbidity (A_{405}) of WAT(1–24) (◆, upper abscissa), a V_L protein (●, lower abscissa), and a BJp (○, lower abscissa) all at 1 mg/ml^{-1} in PBS, pH 7.5, during ASF.

a minicuvette (250 μl maximum volume; Shimadzu, Norcross, GA) and interrogated using a standard bench spectrophotometer (Shimadzu UV160U). The turbidity (optical density) is assessed by measuring the absorbance at 400 nm (Fig. 2). Other wavelengths may be chosen, but the same wavelength should be used consistently throughout the course of the reaction due to the wavelength dependence of scattered light intensity.[15] Generally, fibrillogenesis occurs within 10 days and, considering that a small volume of sample will be lost at every measurement point, the experimenter must determine how often to screen the samples in order to minimize changes in the sample volume over the course of the experiment. A plot of the measured absorbance versus time yields kinetic information for further analysis.[16] The plateau phase at the end point of fibril formation provides only a qualitative value of the fibril content that should only be compared to identical reactions performed in parallel. Due to the relationship between optical density and aggregate structure, this measurement

[15] I. D. Campbell and R. A. Dwek, "Biological Spectroscopy." Benjamin-Cummings, Redwood City, CA, 1984.
[16] M. F. Bishop and F. A. Ferrone, *Biophys. J.* **46**, 631 (1984).

cannot be used as a direct measure of fibril content. Furthermore, other methods, such as ThT fluorescence and electron microscopy, should be employed to confirm that the aggregates are fibrillar.

In addition, quantification of the amount of precursor protein incorporated into fibrils can be achieved indirectly by measuring the free protein concentration and subtracting from the initial. This is accomplished simply by sedimentating fibrillar material at 17,000*g* and 25° for 30 min. Remaining polymers are removed from the resulting supernatant by filtration through a 0.2-μm pore-size, nonadsorbing filter (e.g., Acrodisc, Pall Corp., East Hills, NY). Soluble protein can then be quantified using standard techniques, e.g., HPLC, ε_{280} or colorimetric/fluorescent assays, as appropriate.[17] Centrifugation at 100,000*g* and 25° for 30 min did not significantly alter the concentration of soluble protein in the supernatant when compared to lower speed separations.

Based on the premise that perturbation of a solution of WAT(1–24) peptide is required to induce fibrillogenesis, several other methods have been assessed. Although rigorous in certain cases, and applicable only to peptide fibrillogenesis, they warrant a brief description. Protein concentrations tested using these techniques range between 0.4 and 2 mg/ml suspended in PBS, pH 7.5. The following procedures result in the confirmed fibril formation of WAT peptides within 24 hr: (1) sonication of the solution, delivering 3 W for a 4-sec period every 15 min; (2) continuous stirring of the solution using a magnetic stir bar (kinetics are dependent on the rate of stirring); and (3) manual agitation by repeated pipetting using a p1000 Gilson (Rainin, Woburn, MA). Somewhat intuitively, differences in fibril morphology were observed by electron microscopy; sonication yielding shorter dispersed material and the latter techniques generally result in longer, bundled fibril aggregates.

ASF has proved to be an ideal tool for monitoring fibril formation in systems that require many days to attain equilibrium, such as with whole BJPs; however, the sampling technique can be cumbersome and inconvenient. ASF is well suited for the production of milligram quantities of fibrillar material for use in seeding experiments and structural analyses. Because this method does not rely on extremes of pH or the presence of chaotropic ions to induce fibrillogenesis, it may be considered a better approximation of the physiological paradigm.

Monitoring ASF with in Situ Thioflavin T

Ideally, an *in vitro* fibrillogenesis model should be amenable to rapid and reproducible production of data for subsequent analysis, adhere to

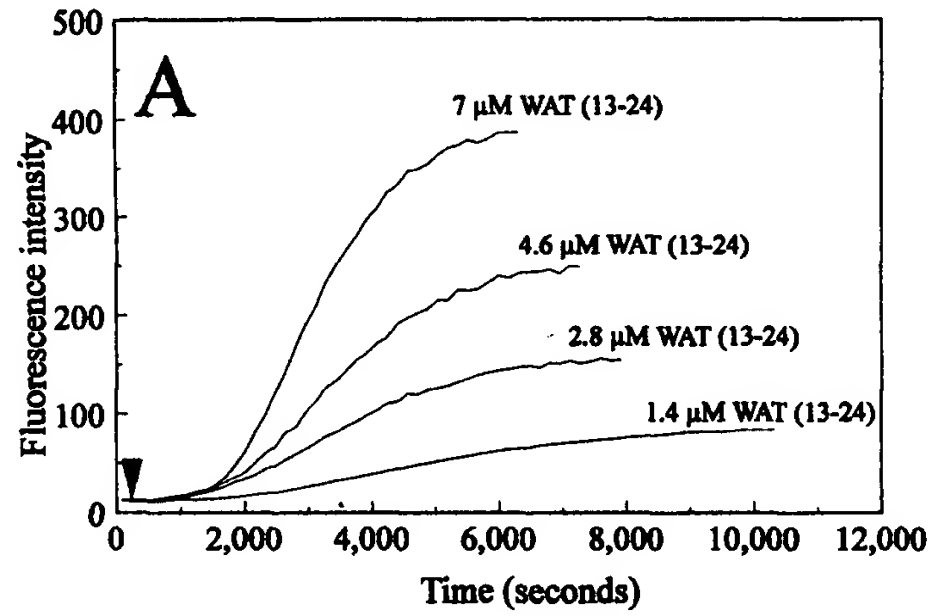
[17] C. M. Stoscheck, *Methods Enzymol.* **182**, 50 (1990).

physiological constraints wherever possible, and be applicable to a wide range of precursor proteins. Methods have been developed from ASF that are capable of monitoring the fibrillogenesis of peptides, IgLC V_L fragments, and BJps using ThT as a reporter of fibril production. This method differs from previously employed ThT-based assays in that the dye is added to the precursor solution *ab initio*. This permits changes in fluorescence intensity at fibril-bound wavelengths to be measured in real time, thus circumventing the need for continuous sampling of the reaction mixture (Fig. 3A). The spectroscopic properties of ThT and its application for detecting and estimating fibril concentration are dealt with in detail elsewhere in this volume.[17a] Suffice to say is that in the presence of amyloid fibrils, ThT fluorescence is enhanced greatly using an excitation wavelength of 450 nm and emission at 490 nm. Furthermore, unbound ThT is essentially nonfluorescent at these wavelengths, a feature that permits its inclusion in the reaction solution without interfering with the measured fluorescence signal. Care should be taken to exclude sulfated macromolecules and other biological polymers, as well as lipid membranes from the solution, as they compete for ThT binding.[18] In addition, nucleic acids and solvents, such as chloroform and dichloromethane, should be excluded as their presence results in ThT fluorescent properties equivalent to the fibril-bound dye.

Before undertaking a systematic investigation of protein fibrillogenesis using this method, important preliminary data must be gathered. Inherent in the application of this technique is the assumption that the inclusion of ThT in the sample mixture will not influence the kinetic or thermodynamic properties of the reaction. This assumption can be validated by performing identical, parallel fibrillogenesis experiments: one reaction followed using *in situ* ThT fluorescence and the second in the absence of ThT, monitored by changes in solution turbidity (Fig. 3B).

Stock solutions of ThT are prepared in HPLC-grade water to a final concentration of 1 m*M*; this solution is then cleared by filtration through a 0.2-μm pore-sized filter. The final concentration may be determined using the Beer–Lambert equation; add 10 μl of stock solution to 0.99 ml of ethanol and measure the absorbance at 416 nm, concentration is calculated using a molar extinction coefficient, ε_{416}, of 26,620 (Sigma product information). The concentration should be determined routinely to compensate for changes due to evaporation or loss of material in subsequent filtrations. The stock solution can be kept for over 12 months without loss of amyloid-bound fluorescence intensity.

[17a] H. LeVine, *Methods Enzymol.* **309** [18] (1999) (this volume).
[18] M. T. Elghetany and A. Saleem, *Stain Technol.* **63,** 201 (1988).



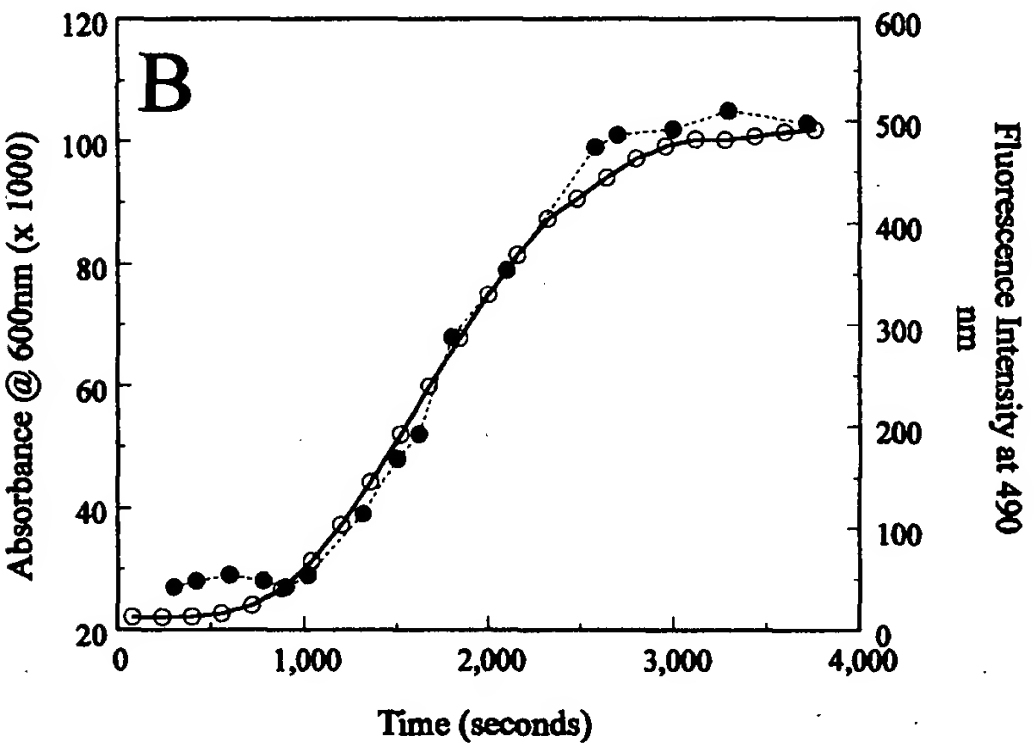


FIG. 3. Analysis of WAT(13–24) fibrillogenesis using *in situ* ThT. (A) *In situ* ThT fibrillogenesis of increasing concentrations of WAT(13–24). Arrow indicates addition of protein. (B) Comparison of WAT(13–24) fibril formation using *in situ* ThT (○, right ordinate) and turbidity (●, left ordinate). Fluorescence measurements were taken using an SLM Aminco Bowman Series II as described.

ASF is monitored using *in situ* ThT using an SLM Aminco-Bowman Series II spectrofluorometer with thermostatted cuvette housing and stirring capabilities. Excitation and emission wavelengths are 450 and 490 nm, respectively, with corresponding slit widths of 4 and 8 nm. Data are collected

at 25°, over 8 hr, in "time trace" mode and a frequency of one data point per second. The fluorescence signal is corrected internally for lamp drift and pulsing by division by a reference PMT signal. Precursor proteins are prepared as described in the ASF technique at 1 mg/ml^{-1} in PBS, pH 7.5. Immediately prior to the start of the reaction, stocks of precursor and ThT are diluted into filtered PBS, pH 7.5, in a 4-ml volume fluorimetric cuvette. The solution is mixed by inversion and analyzed. Agitation is provided by inclusion of a microstir bar (7 × 2 mm). The importance of the mixing speed is discussed later. The precise micromolar concentrations of protein and ThT vary depending on the nature of the protein to be studied, generally 1–10 μM protein and 2–20 μM ThT are employed. A twofold molar excess of ThT is used to ensure saturation of the binding sites. The precise quantities should be determined by the experimenter. Care should be taken not to use much greater than saturating concentrations of the dye as it significantly self-quenches at these levels and sensitivity will be compromised.

The inclusion of ThT in the reaction mixture has several advantages over previous sampling methodologies. Most notable is the enhanced fluorescence intensity of the sample with respect to the addition of ThT to an equivalent sample, postfibril formation. This phenomenon consistently results in a two- to eightfold increase in the fluorescence intensity of a synthetic fibril preparation at equilibrium (end point), formed in the presence of ThT, relative to a parallel solution into which ThT is titrated to saturation postfibril formation (Fig. 4). Preliminary data suggest that this results from the inaccessibility of many ThT-binding sites at the reaction end point, perhaps due to the organization of ThT-positive filaments into fibers and higher order macrocomplexes.

Agitation-Independent Fibrillogenesis of Recombinant V_L Proteins

An attempt to construct a unified theory to rationalize the fibrillogenic potential of IgLCs and their fragments has led to an evaluation of the thermodynamic stability of proteins and its correlation with the propensity to form aggregates. This has initiated a search for destabilizing amino acid substitutions that may confer to the protein, an inherent propensity to aggregate.[19,20] Based on this postulate, a rapid reproducible method of fibrillogenesis has been developed and applied, as in previous systems, to

[19] R. Wetzel, *Adv. Prot. Chem.* **50,** 183 (1997).
[20] M. R. Hurle, L. R. Helms, L. Li, W. Chan, and R. Wetzel, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 5446 (1994).

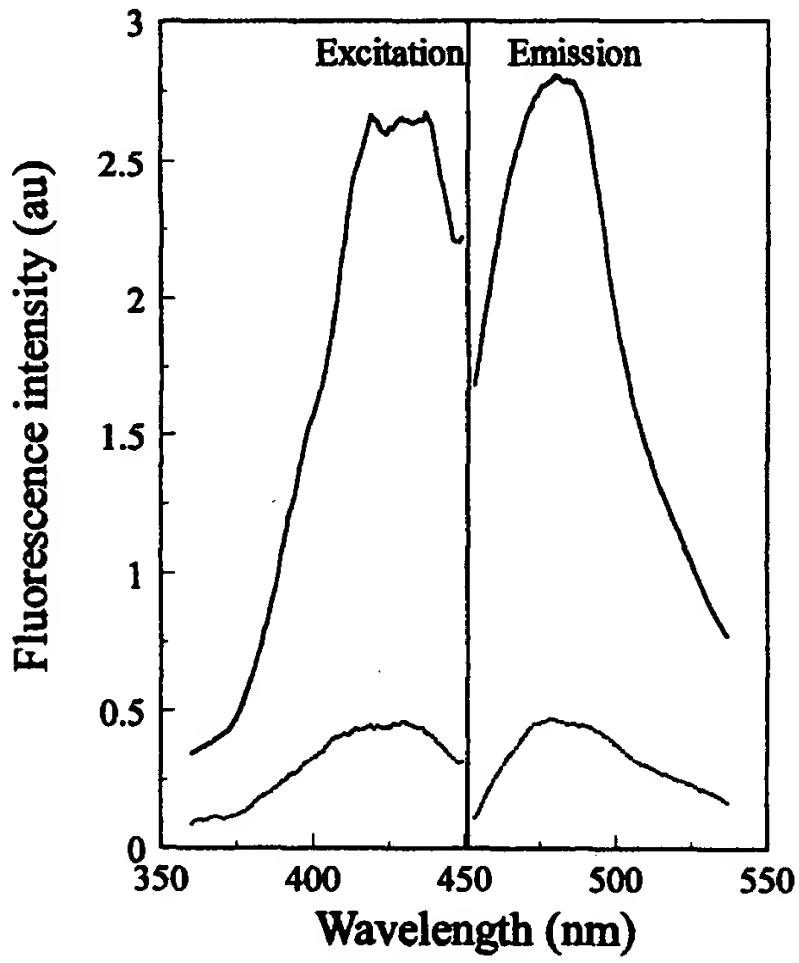


FIG. 4. Comparison of ThT fluorescence sensitivity. Excitation and emission spectra of ThT when added *ab initio* to the fibrillogenesis of WAT(13–24) (solid) and titrated to saturation postfibril formation (dotted).

recombinant V_L fragments.[20,21] The principle underlying this fibrillogenesis assay is that temperature-induced destabilization of the precursor protein results in fibril formation. The assay is performed essentially as that described for agitation-induced fibrillogenesis in the fluorimeter. The major difference is that agitation is no longer required. A stock solution of V_L protein at 1 mg/ml^{-1} (approximately 90 μM) is filtered through a 0.2-μm pore-sized filter immediately prior to polymerization. The A_{280} is measured and the protein concentration is reconfirmed by application of the Beer–Lambert equation using the appropriate molar extinction coefficient. The fibrillogenesis reaction mixture consists of 3 μM V_L protein and 6 μM filtered ThT. This is prepared by dilution of the protein stock solution into PBS, pH 7.5, at 37° to yield a final volume of 3 ml. A microstir bar is included at a constant stirring speed of one to two revolutions per second

[21] P. W. Stevens, R. Raffen, D. K. Hanson, Y.-L. Deng, M. Berrios-Hammond, F. A. Westholm, C. Murphy, M. Eulitz, R. Wetzel, M. Schiffer, and F. J. Stevens, *Prot. Sci.* **4**, 421 (1995).

to prevent the settling of precipitating material. Fibrillogenesis is performed at 37° and is modeled using the heterogeneous nucleation model of Bishop and Ferrone[16] (Fig. 5). Higher temperatures result in a decrease in lag phase concomitant with an increase in the rate of hyperbolic fibril growth. This technique has been applied successfully to distinguish an amyloidogenic λ VI V_L from a cast-forming λ VI V_L based on the rates of fibrillogenesis at 37°.

Phenomenology

When ASF is performed in an orbital shaker, certain technical nuances need to be considered. The first is precautionary; namely, that the reaction mixtures should contain 0.02% NaN_3 as a preservative. Its addition, however, results in a high absorbance at 230 nm that will prevent turbidity measurements using UV wavelengths and preclude the use of spectrophotometric protein determination assays using this wavelength. Second, because the samples are incubated over periods of days at 37°, the tubes should be stoppered securely and held in place with Parafilm to prevent evaporation that would result in changes in protein concentration. Although not as widely available as bench-top spectrophotometers, instruments are available commercially to measure absorbance in 1-cm-diameter test tubes; the Spectronic 20 Genesys is one example (Spectronic, Rochester, NY). This

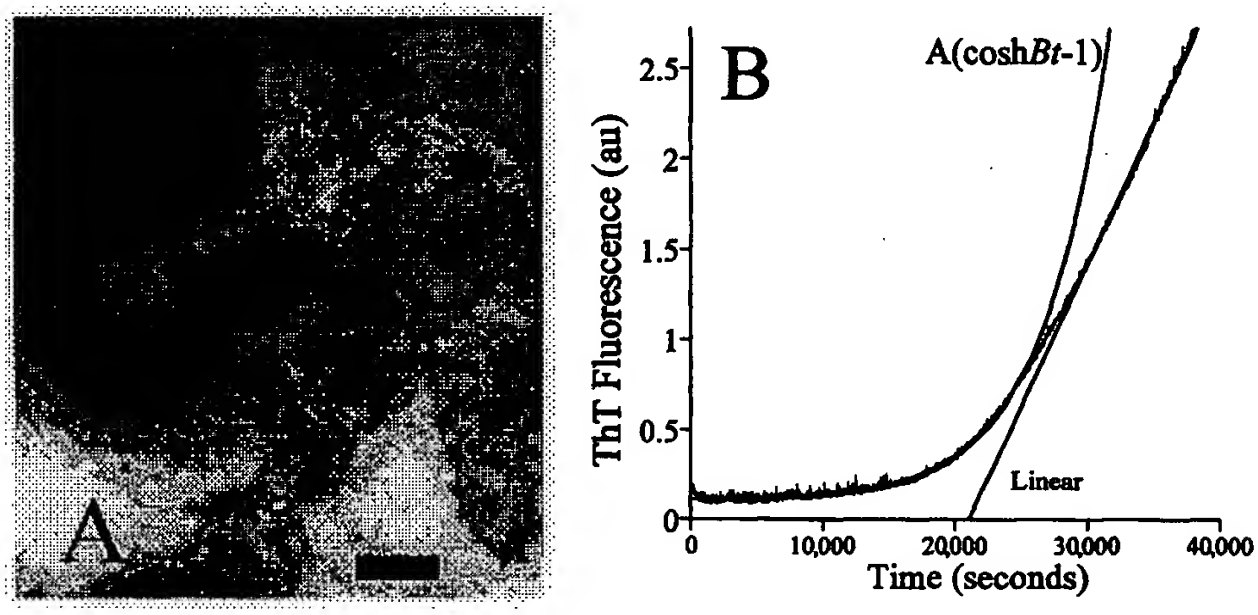


FIG. 5. Fibrillogenesis of V_L proteins at 37°. (A) Negatively stained, electron microscopy of V_L fibrils formed by fibrillogenesis at 37°. Bar: 300 nm. (B) Kinetics of V_L fibril formation. Least-squares analysis of data using A(cosh *Bt*-1) according to Bishop and Ferrone[16] provides excellent agreement with the first 10% of the data, as predicted, indicative of nucleation-dependent polymerization. Linear extrapolation of the later aggregation curve yields a value for the lag phase, i.e., 22,000 sec.

instrument would preclude the need for sampling the reaction mixture and obviate many of the aforementioned problems. Finally, the orientation of the tubes in the orbital shaker is critically important. This is believed to relate to the extent of agitation achieved. Samples placed upright mix vortically, which generally results in little or no fibrillogenesis. When placed at a 45° angle, however, mixing is erratic; this motion results in optimal fibril formation.

At room temperature without agitation, IgLC peptides, V_L, and Bence Jones proteins do not form fibrils. The process is initiated in the fluorimetric cuvette by inclusion of a microstir bar, and the sample is agitated rather than vortex-mixed, somewhat analogous to the importance of tube angle in ASF. This is achieved by setting the stirrer speed such that the stir bar "jumps" in the cuvette. Although somewhat anecdotal, the importance of the motion of the stir bar cannot be understated. When V_L fragments and BJPs are mixed vortically, the predominant aggregates are amorphous with none of the microscopic or tinctorial properties of fibrils. However, when the same protein is agitated, fibrils dominate (Fig. 6).

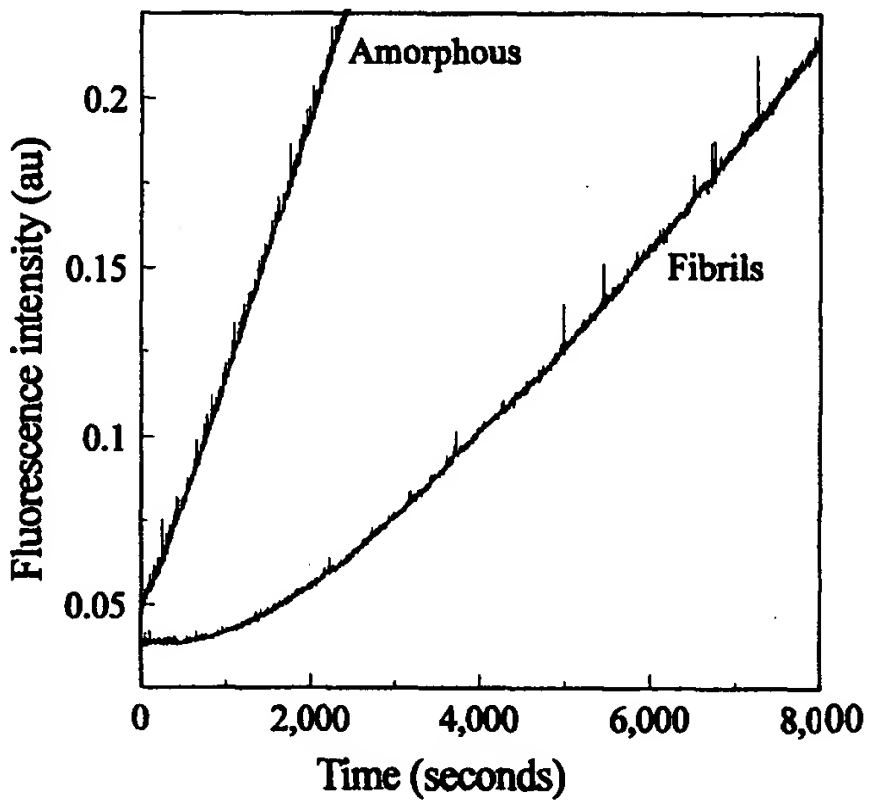


FIG. 6. Fibrillogenesis in the cuvette. Vortex mixing vs agitation. Amorphous precipitates arise when the solution is mixed vortically, which results in a linear increase in the intensity of scattered light. Fibrils are formed when the stir bar motion is erratic and the solution agitated. Both samples contain 3 μM BJp and 10 μM ThT in 3 ml of PBS, pH 7.5, and 25°. Emission data were collected using an excitation of 450 nm. The PMT voltage and slit widths were identical for both samples.

Quartz cuvettes should not be used to monitor fibril formation in the fluorimeter. Fibrillogenesis of IgLC proteins has not been observed within 72 hr using a quartz cuvette. Polystyrene cuvettes (Starstedt, Newton, NC) yield rapid reproducible results and are used routinely in our laboratory. Polymethacrylate cuvettes (Sigma) are less amenable to fibril formation, observed as an increase in the lag time with respect to fibrillogenesis performed in polystyrene cuvettes. This result remains enigmatic, but probably reflects the role that surface adhesion plays in the aggregation of these proteins.

Sampling versus in Situ ThT Monitoring

In many instances when sampling reaction mixtures, an aliquot is diluted into a larger volume followed by titration with ThT to measure fibril content. This approach results in a rapid, exponential decrease in the fluorescence intensity of the ThT within 5 min of dilution and has been witnessed with synthetic fibrils prepared from IgLC peptides, V_L proteins, Aβ(25–35), and Aβ(1–40) alike. The phenomenon is under investigation, but may be related to reequilibration of the monomer, polymer, or polymeric aggregates.

When synthetic V_L and Aβ(25–35) fibrils, formed in the presence of 10 μM ThT, are diluted into a solution of 10 μM ThT, the decrease in the fluorescence intensity is again observed, suggesting that ThT dissociation from the fibrils is not a factor. Whatever the mechanism, there is clearly a decrease in the fluorescence intensity. The inclusion of ThT within the polymerization reaction, therefore, precludes the need for reaction mixture sampling and circumvents the sampling problems associated with this phenomenon.

Summary

These techniques permit the production of bulk quantities of fibrils and provide methods for monitoring the kinetics of fibrillogenesis. Experiments performed in the fluorimeter require low protein concentrations, sampling is not necessary (with ThT *in situ*), and the measured fluorescence signal is indicative of fibril content and is not complicated by the presence of amorphous aggregates. However, ASF using the orbital shaker is a simple, rapid, initial procedure, adequate for screening for fibrillogenic potential, in which multiple experiments can be performed simultaneously and over long periods of incubation. These methods may be used to investigate the fibrillogenesis of V_L proteins and BJps as a means of predicting pathogenicity, as well as providing information on the basic biophysical principles underlying light chain aggregation.

Acknowledgments

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[15] Inhibition of Aggregation Side Reactions during *in Vitro* Protein Folding

By ELIANA DE BERNARDEZ CLARK, ELISABETH SCHWARZ, and RAINER RUDOLPH

When synthetic or natural genes or cDNAs are overexpressed in the cytosol of microbial host cells such as *Escherichia coli,* recombinant proteins can be produced in large amounts. Although high-level expression is usually achieved using standard recombinant DNA techniques, the polypeptides are often sequestered in the form of insoluble, inactive inclusion bodies. These large, dense particles often span the whole diameter of the host cell. After proper isolation they consist primarily of the recombinant protein. Active protein can be recovered from the inclusion bodies by solubilization in chaotrophic buffer systems and subsequent *in vitro* folding. However, unproductive side reactions (predominantly aggregation) often compete with correct folding during *in vitro* folding. Various techniques, some of which are summarized in this article, have been developed to inhibit aggregation side reactions and to ensure efficient *in vitro* protein folding. These methods, which can now be considered as standard laboratory techniques, allow the refolding of many inclusion body proteins on the laboratory scale or even in industrial production processes.

Isolation of Inclusion Bodies

Many different protocols for inclusion body isolation are described in the literature. Generally, inclusion bodies are harvested by centrifugation after cell lysis.[1] The quality of the inclusion bodies can be improved considerably before centrifugation by maximum cell lysis and dissociation of all particulate matter. Maximum cell disruption can be achieved by combining

[1] R. Rudolph, G. Böhm, H. Lilie, and R. Jaenicke, *in* "Protein Function" (T. E. Creighton, ed.), p. 57. IRL Oxford Univ. Press, Oxford, 1997.

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